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**This is a pre print version of the following article:**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/153169> since 2018-09-19T10:02:33Z

*Published version:*

DOI:10.1210/jc.2014-3636

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**A case of severe hyperaldosteronism caused by a de novo mutation affecting a critical ‘salt bridge’ Kir3.4 residue.**

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**Short title:** de novo *KCNJ5* mutation causing hyperaldosteronism.

**Key words:** primary aldosteronism, *KCNJ5* mutations, familial hyperaldosteronism, Kir3.4

**Words count** (excluding abstract, figure captions, acknowledgements and references): 1800.

**Number of figures:** 2 (2 in colours) + 2 Supplemental Figures

**Grant Support:** S.M. was supported in 2013 by a fellowship from the Società Italiana dell’Ipertensione Arteriosa; P.M. is in receipt of a grant from the Italian Ministry of the Instruction, University and Research (grant ex-60%-2013). S.B. was supported by Deutsche Forschungsgemeinschaft (FOR 1086). This work was supported in part by the Intramural Program of the *Eunice Kennedy Shriver* National Institute of Child Health & Human Development (NICHD), National Institutes of Health (NIH), Bethesda, MD 20892, USA.

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**Authors disclosure/conflict of interest:** the authors have nothing to disclose.

## Abstract

**Context.** Familial hyperaldosteronism type III (FH-III) is a rare and clinically heterogeneous condition, that can display mild as well as severe phenotypes. Point mutations in the *KCNJ5* gene, affecting the ion selectivity of the inward rectifier K<sup>+</sup> channel 4 (Kir3.4), underlie the molecular basis of FH-III.

**Objective.** To investigate the effects of a *de novo* germline *KCNJ5* mutation.

**Patients and Methods.** We describe the case of a girl, who came to medical attention at the age of two years because of polydipsia, polyuria and failure to thrive. The patient, affected by hypertension and hypokalemia, was diagnosed with primary aldosteronism on the basis of extremely high aldosterone levels and suppressed plasma renin activity. Genomic DNA was isolated and *KCNJ5* sequenced. Human adrenocortical cells were used as an *in vitro* model for the functional characterization of the mutant channel.

**Results.** *KCNJ5* sequencing in the index case and her parents revealed a *de novo* p.Glu145Gln germline mutation. The substitution resulted in Na<sup>+</sup>-dependent depolarization of adrenal cells and increased intracellular calcium concentration, which activated the transcription of *NR4A2* and, in turn, *CYP11B2*. Pharmacological studies revealed that the mutant channel was insensitive to tertiapin-Q and calcium-channel blocker verapamil.

**Conclusions.** Herein we report the identification of a novel *KCNJ5* germline mutation responsible for severe hyperaldosteronism that presented in infancy with symptoms of diabetes insipidus. The findings of this study further elucidate the etiology of FH-III and expand our knowledge of this rare condition.

## INTRODUCTION

Primary aldosteronism (PA) is the most frequent cause of secondary hypertension(1). In addition to the predominant sporadic forms of PA there are at least three distinct familial variants, named familial hyperaldosteronism (FH) types I, II and III. FH-III is an autosomal dominant disease clinically distinct from the first two familial forms(2) caused by heterozygous mutations in the *KCNJ5* gene encoding the G protein-activated inward rectifier K<sup>+</sup> channel 4 (Kir3.4)(3). So far, five germline mutations in the *KCNJ5* gene have been identified and functionally characterized in patients with FH-III(4,5). All these missense mutations are located near or within the conserved glycine-tyrosine-glycine (GYG) motif of the selectivity filter of the channel. Here we report and characterize a germline missense mutation affecting another conserved residue located N-terminal to the GlyTyrGly signature sequence. This substitution, p.Glu145Gln, previously reported as somatic mutation in sporadic APAs(6), was identified in a patient with severe hyperaldosteronism that presented in infancy. Interestingly, the importance of this p.Glu145Gln substitution for Kir3.4 function had been predicted and confirmed *in vitro*(7) a decade before the *KCNJ5* mutations were linked to FH-III.

## MATERIALS AND METHODS

Detailed Materials and Methods are available on The Endocrine Society's Journals Online web site at <http://jcem.endojournals.org>.

## RESULTS

### Case report

The index case is a Caucasian girl, born to nonconsanguineous parents. At the age of 8 weeks poor weight gain was noticed. At the age of 8 months, she displayed failure to thrive, polydipsia and polyuria. At the age of 2 years, her height was 80 cm (SDS=-2.1), weight 12 kg (SDS=-2.7), blood pressure 115/65 mmHg (SBP > 99<sup>th</sup> height-corrected percentile for age). The urinary output was 3 L/day, urine specific gravity ranged 1000-1003, serum potassium was 2.3 mmol/L, sodium 140 mmol/l. PA was diagnosed on the basis of extremely elevated serum aldosterone (>5000 pmol/L, normal range 111-830 pmol/L) and suppressed PRA (0.2 ng/mL/h, normal range, 0.98-4.18). Aldosterone remained elevated (>5000 pmol/L) after dexamethasone suppression test (4 ug/day for 2 days). Treatment with spironolactone (50 mg/day) and potassium supplements were started.

At the age of 4 years no difference in the aldosterone/PRA ratio before (>2632) and after (>2849) orthostatic exercise was observed. CT and MRI failed to reveal adrenal masses. At the age of 8 years, the patient was admitted to the Warren Magnuson Clinical Center (National Institutes of Health, NIH, Bethesda, Maryland). At admission she was off spironolactone for 2 weeks. Her height was 134.7 cm (75<sup>th</sup> percentile), weight 34.4 kg (90<sup>th</sup> percentile) and pubertal stage Tanner B2P2. She was hypertensive (135/100 mm Hg, >99<sup>th</sup> height-corrected percentile for age) and serum potassium was 2.3 mmol/L. Serum creatinine was 0.6 mg/dL (0.7-1.33 mg/dL). Serum aldosterone was 8365 pmol/L, PRA 0.6 ng/mL/h and serum 18OH-corticosterone 27.0 nmol/L (0.2-2.3). Intravenous saline loading test confirmed non-suppressibility of aldosterone production (8815 pmol/L after saline infusion). A 2-day dexamethasone suppression test showed a decrease of serum aldosterone from 16537 to 9086 pmol/L excluding glucocorticoid-remediable aldosteronism. Adrenal vein sampling was performed displaying bilateral aldosterone overproduction.

Subsequently, she has been treated with spironolactone (50-400 mg/day), potassium chloride and calcium-channel blockers. She remained hypertensive and hypokalemic (serum potassium 2.7-3.9 mmol/L). The patient underwent pubertal development started at 11 years and progressed to Tanner stage B4P4 but no menarche occurred. Because of the low effectiveness of the therapy, bilateral adrenalectomy was recommended, but the family denied the consent.

At the age of 19 she was re-evaluated at NIH. She was on four antihypertensive medications and potassium supplements; serum creatinine was 2.19 mg/dL, potassium 3.2 mmol/L and urine protein/creatinine ratio 2.5 mg/mg. Transthoracic echocardiography revealed mildly dilated aortic root and ascending aorta without left ventricular hypertrophy. The patient consented to bilateral adrenalectomy which was performed laparoscopically (Supplemental Figure S1).

### **Identification of a novel *KCNJ5* mutation**

Sequencing of the *KCNJ5* gene from peripheral blood DNA identified a novel germline heterozygous c.433G>C substitution (Figure 1A), that causes a glutamic acid to glutamine substitution at position 145 (p.Glu145Gln). The mutation was absent from the parents peripheral blood DNA (Figure 1A-B), thereby indicating a *de novo* mutation in our patient. An amino acid alignment of Kir3.4 showed that Glu145 is highly conserved among orthologs and paralogs (Supplemental Figure S2).

### **Gene expression study in HAC15 cells**

To define the effect of *KCNJ5* c.433G>C substitution in adrenal cell function the mutant channel p.Glu145Gln was overexpressed in HAC15 cells and gene expression evaluated by real-time PCR. We observed a significant up-regulation of both *CYP11B2* ( $2.4 \pm 0.5$  fold;  $P < 0.001$ ) and its transcriptional regulator *NR4A2* ( $60.8 \pm 13$  fold;  $P < 0.001$ ) in Kir3.4 p.Glu145Gln overexpressing cells, compared to cells expressing the wild-type protein (Figure 1C).

### **Na<sup>+</sup>-dependent depolarization of adrenal cells expressing Kir3.4 p.Glu145Gln**

Whole cell currents in NCI-H295R cells expressing wild-type Kir3.4 were very small and did not change after removal of  $\text{Na}^+$  from the extracellular solution (Fig. 2A). In contrast, cells expressing Kir3.4 p.Glu145Gln showed larger inward and outward currents (Fig. 2A) under control conditions compared to *KCNJ5*<sup>WT</sup>-transfected cells. The inward current was reduced under  $\text{Na}^+$ -free conditions demonstrating the  $\text{Na}^+$  permeability of the mutant channel. Accordingly, mutant channel expressing cells were strongly depolarized under resting conditions, and hyperpolarized under  $\text{Na}^+$ -free conditions (Fig. 2B).

### **Pharmacology of Kir3.4 p.Glu145Gln**

Kir3.4 is activated by G-protein-coupled receptors and by high intracellular  $\text{Na}^+$  concentrations (8). Accordingly, an inward current was activated in cells expressing wild-type Kir3.4 when 30 mM  $\text{Na}^+$  and 0.5 mM GTP were present in the pipette solution, and when extracellular  $\text{K}^+$  was increased to 50 mM. Under these conditions, the inward current in *KCNJ5*<sup>WT</sup> transfected cells was inhibited by application of 1  $\mu\text{M}$  tertiapin-Q(9) to the bath (Fig. 2C). The mutant p.Glu145Gln channel was insensitive to tertiapin-Q (Fig. 2C). The L-type  $\text{Ca}^{2+}$ -channel blocker verapamil, previously shown to inhibit other Kir3.4 mutants(10) had no significant effect on Kir3.4 p.Glu145Gln ( $81 \pm 10\%$  remaining inward current,  $p=0.25$ ,  $n=10$ ).

### **Kir3.4 p.Glu145Gln increased cytosolic $\text{Ca}^{2+}$ levels in adrenal cells.**

Under control conditions,  $\text{Ca}^{2+}$  concentration was increased in NCI-H295R cells expressing the mutant channel, compared to cells expressing wild-type Kir3.4 (Fig. 2D). In  $\text{Na}^+$ -free extracellular solution (a condition where the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger, NCX, is inhibited or even works in reversed mode),  $\text{Ca}^{2+}$  concentration was further increased in cells expressing Kir3.4 p.Glu145Gln. Application of verapamil under control conditions led to a reduction of  $\text{Ca}^{2+}$  concentration in cells expressing the mutant, although  $\text{Ca}^{2+}$  was not normalized to wild-type levels (Fig. 2D). Stimulation of cells with 15 mM  $\text{K}^+$  increased  $\text{Ca}^{2+}$  in wild-type Kir3.4 expressing cells, but had a minor effect on cells expressing the p.Glu145Gln channel, mainly because the mutant expressing cells were already strongly depolarized under control conditions (Fig. 2B). Calcium increase in wild-type Kir3.4 expressing cells was smaller than in untransfected cells,



indicating that the wild-type channel was activated under these conditions, therefore reducing depolarization and subsequent activation of voltage-activated  $\text{Ca}^{2+}$  channels. The effect of stimulation with angiotensin II was similar in wild-type and mutant *KCNJ5* transfected cells (Fig. 2D).

## DISCUSSION

An important breakthrough in the understanding of the molecular mechanisms underlying autonomous aldosterone production in PA has been made recently by the discovery of germline and somatic mutations in genes that affecting calcium entry in adrenal zona glomerulosa cells(3,11,12). *KCNJ5* gene mutations, which cause both FH-III and a consistent proportion of sporadic APAs(3,4), impair the selectivity filter of the Kir3.4 K<sup>+</sup> channel, leading to angiotensin II -independent depolarization of adrenal cells and constitutive aldosterone production.

Ion selectivity of all K<sup>+</sup> channels (including Kir3.4) was shown to be dependent on the presence of specific amino acids within the P-loop domain(13). In addition to the GlyTyrGly motif, that coordinates dehydrated K<sup>+</sup> ions as they move through the channel(14), further structural features of the channels are necessary to confer K<sup>+</sup> selectivity(15). Yang et al. demonstrated that two oppositely charged conserved residues in Kir2.1, Glu138 and Arg148, could form a salt bridge(16) and subsequently Dibb et al. mutated the equivalent glutamate residue in Kir3.4, Glu145, and confirmed that the p.Glu145Gln substitution resulted in loss of ion selectivity and inward rectification(7).

The p.Glu145Gln mutation was first reported by Åkerström et al. as a somatic mutation in two APAs(6). Herein we describe the first case of FH-III associated with this molecular defect and characterize the functional consequences of the mutation in adrenal cells. Electrophysiological studies using adrenocortical NCI-H295R cells revealed that loss of ion selectivity of the p.Glu145Gln mutated channel led to cell membrane depolarization as observed in *Xenopus* oocytes(7). Ca<sup>2+</sup> measurements in adrenocortical cells disclosed pathologically increased cytosolic Ca<sup>2+</sup> levels, which were further increased upon removal of extracellular Na<sup>+</sup>. The latter Ca<sup>2+</sup> increase was likely caused by the reversed mode of NCX that was promoted by Na<sup>+</sup> loading of the cells. Besides the activation of voltage-gated Ca<sup>2+</sup> channels and the Ca<sup>2+</sup> influx through the mutated Kir3.4 channel itself(17), impaired NXC-mediated Ca<sup>2+</sup> extrusion or even reversed transport mode could account for the severe phenotype of cells expressing mutant Kir3.4. Taken together, the effects of the p.Glu145Gln mutant expressed in adrenal cells were reminiscent to those reported for other, mostly somatic mutations of Kir3.4(10,18) Interestingly, expression of the Kir3.4 wild-type channel diminished the Ca<sup>2+</sup> signal provoked by high K<sup>+</sup> indicating a physiological function of Kir3.4 in limiting overwhelming aldosterone secretion rather than setting the baseline activity of glomerulosa cells.

In accordance with an increase in  $\text{Ca}^{2+}$  influx, expression of the mutated channel in HAC15 adrenal cells increased *NR4A2* and *CYP11B2* transcript levels compared to wild type *KCNJ5* expressing cells, supporting a mechanistic role for this mutation in the activation of aldosterone production.

Based on the limited number of cases reported to date, the clinical severity of FH-III varies significantly, ranging from early to late manifestations with mild to severe hypertension and different responsiveness to medical therapy(4,5). So far it is not clear whether a genotype-phenotype correlation exists in FH-III. The patient described herein is one of the most severe cases reported so far. The girl presented with severe hyperaldosteronism in the first months of life with profound hypokalemia mimicking nephrogenic diabetes insipidus. Interestingly, in spite of the extreme hyperaldosteronism and hypokalemia, blood pressure was only slightly elevated. This is in agreement with other reports of congenital mineralocorticoid excess in children, showing that overt hypertension is not uniformly present in younger patients(19,20).

Treatment with large doses of spironolactone and potassium supplementation in our patient was only partially effective. Addition of calcium channel blockers did not result in a significant improvement and had no effect on plasma aldosterone concentration in agreement with the experimental data showing no effect of the L-type calcium-channel blocker verapamil on the mutated channel.

In conclusion, we report the identification of a novel *KCNJ5* germline mutation responsible for severe hyperaldosteronism that presented in infancy with symptoms of diabetes insipidus. This p.Glu145Gln substitution, as for other *KCNJ5* mutations, causes  $\text{Na}^{+}$  dependent cell membrane depolarization and disturbed intracellular  $\text{Ca}^{2+}$  homeostasis in adrenocortical cells. Indeed, the findings of this study further elucidate the etiology of FH-III and expand our knowledge of this rare condition.

### **Acknowledgements.**

We thank Christina Sterner (Medical Cell Biology, University of Regensburg, Germany) for her expert assistance. We acknowledge the support by the Intramural Program of the Eunice Kennedy Shriver National Institute of Child Health & Human Development (NICHD), National Institutes of Health (NIH), and its staff (Elena Belyavskaya, MD and Charalambos Lyssikatos, among others).

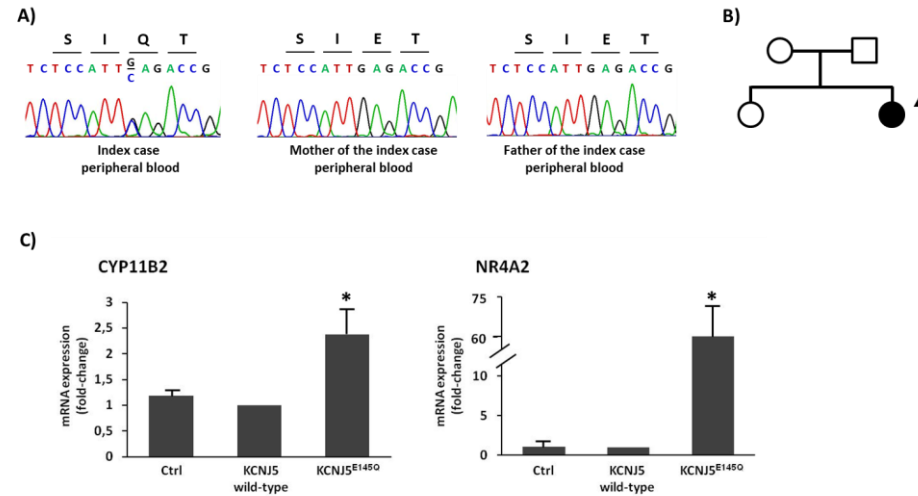
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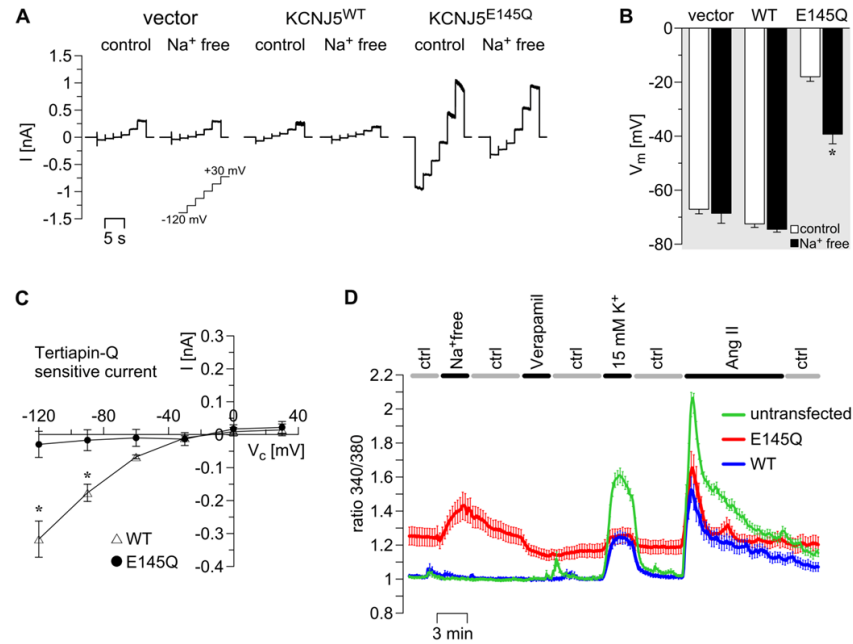
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## Figures.



**Figure 1.** (A) Sequencing of peripheral blood DNA showing the *KCNJ5* c.433G>C substitution resulting in the p.Glu145Gln (p.E145Q) mutation in the index case, but not in the parents. (B) Pedigree of kindred with germline *KCNJ5* mutation; affected individual is shown as filled symbol. (C) *CYP11B2* and *NR4A2* gene expression study in HAC15 adrenocortical cells overexpressing empty expression vector (Ctrl) or human wild-type *KCNJ5* (*KCNJ5* wild-type) or *KCNJ5* cDNA encoding the p.Glu145Gln substitution (*KCNJ5*<sup>E145Q</sup>). Gene expression levels were quantified by TaqMan real-time PCR using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as endogenous control. Each bar represents the mean  $\pm$  SD of relative fold change (over *KCNJ5* wild-type) of gene expression in four independent experiments. Each assay was performed in triplicate. \*, P<0.05 compared to *KCNJ5* wild-type. CYP11B2 = cytochrome P450, family 11, subfamily B, polypeptide 2; NR4A2 = nuclear receptor subfamily 4, group A, member 2.



**Figure 2.** The Kir3.4 p.Glu145Gln mutation induced a Na<sup>+</sup> influx and increased intracellular Ca<sup>2+</sup> levels in adrenal NCI-H295R cells. Representative whole cell currents (**A**) and membrane voltage (**B**) of NCI-H295R cells transfected with empty vector (n=5), wild-type *KCNJ5* (WT, n=10), or mutant *KCNJ5* encoding the p.Glu145Gln substitution (E145Q, n=16). Currents and voltage were measured under control (ctrl, normal Ringer solution) and Na<sup>+</sup>-free (using N-methyl-D-glucamine chloride, NMDG<sup>+</sup>, instead of NaCl) conditions. (**C**) The tertiapin-Q (1  $\mu$ M) sensitive current of cells expressing the *KCNJ5* wild-type (WT; n=12) or mutant *KCNJ5* encoding the p.Glu145Gln substitution (E145Q; n=6) is shown. To stimulate currents in cells expressing the wild-type channel, the pipette solution was supplemented with Na<sup>+</sup> (30 mM) and GTP (0.5 mM) and bath K<sup>+</sup> was increased (50 mM K<sup>+</sup>). (**D**) Fura-2 Ca<sup>2+</sup> measurements: Traces show mean values of 340 nm/380 nm ratios  $\pm$



SEM as a measure of intracellular  $\text{Ca}^{2+}$  concentration (WT in black, E145Q in red, and untransfected cells in green; n=17-26 per group) under control and  $\text{Na}^+$  free conditions. Verapamil (10  $\mu\text{M}$ ) and angiotensin II (Ang II, 20 nM) were applied in control solution. In the solution containing 15 mM  $\text{K}^+$ , the  $\text{Na}^+$  concentration was reduced to the same extent. \* =  $p < 0.05$  comparing control and  $\text{Na}^+$ -free (B), or wild-type and Kir3.4 p.Glu145Gln (D) expressing cells.

**A case of severe hyperaldosteronism caused by a de novo mutation affecting a critical ‘salt bridge’ Kir3.4 residue.**

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**Grant Support:** S.M. was supported in 2013 by a fellowship from the Società Italiana dell’Ipertensione Arteriosa; P.M. is in receipt of a grant from the Italian Ministry of the Instruction, University and Research (grant ex-60%-2013). S.B. was supported by Deutsche Forschungsgemeinschaft (FOR 1086). This work was supported in part by the Intramural Program of the *Eunice Kennedy Shriver* National Institute of Child Health & Human Development (NICHD), National Institutes of Health (NIH), Bethesda, MD 20892, USA.

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**Authors disclosure/conflict of interest:** the authors have nothing to disclose.

## Supplementary file

### Materials and methods

#### *DNA analysis.*

The study was approved by the institutional review board, and the patient and her parents gave informed consent for DNA analysis. Genomic DNA was extracted from peripheral leukocytes by standard procedure. The coding sequence of *KCNJ5* gene was amplified by PCR in two fragments and the amplification products were purified and directly sequenced using automated DNA sequencer (3130 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA). The sequences of primers are available on request. Genbank cDNA entry with accession number NM\_000890.3 was used as a reference sequence.

#### *Cell culture and transfection*

For gene expression studies HAC15 human adrenocortical carcinoma cells were cultured in Dulbecco's Modified Eagle/F12 medium (Invitrogen, Carlsband, CA) supplemented with 10% Cosmic Calf Serum (HyClone, Logan, UT), 1% insulin/transferrin/selenium Premix (BD Biosciences, Sparks, MD) and antibiotics.

HAC15 cells were electroporated using the Amaxa electroporator (program X005, Amaxa Biosystems, Cologne, Germany) in 100  $\mu$ L of nucleofector solution R. After electroporation, cells were plated in 6-well plates with 5 mL of growth medium/well and allowed to recover for 24 h, then starved overnight in 0.1% low serum media (0.1% Cosmic calf serum and antibiotics).

For electrophysiological studies adrenocortical carcinoma NCI-H295R cells (CLS, Eppelheim, Germany) were cultured in a commercial cell-line specific DMEM/F12 medium containing: 15 mM HEPES, 6.25  $\mu$ g/mL insulin, 6.25  $\mu$ g/mL transferrin, 6.25 ng/mL selenium, 1.25 mg/mL bovine serum albumin, 5.35 mg/mL linoleic acid, and 2.5% Nu-Serum I (MG-42, CLS, Eppelheim, Germany). The cells were maintained at 37°C under a humid atmosphere of 95% air/5% CO<sub>2</sub>. Plasmids (pMA-RQ (ampR)) containing the cDNA sequence of wildtype human *KCNJ5* and *KCNJ3* were purchased from Invitrogen/Geneart. For expression in mammalian cells, cDNAs were subcloned into the bicistronic expression vector pIRES-CD8(1). The plasmid containing the cDNA sequence of mutated *KCNJ5*<sup>E145Q</sup> was generated by site-directed

mutagenesis according to standard protocols. One day before the experiment,  $1 \times 10^6$  cells were transfected with 5  $\mu$ g of wildtype *KCNJ5* or mutant *KCNJ5* containing pIRES-CD8 plasmids, or with the empty vector using an electroporation system (NEON, Life Technologies GmbH, Darmstadt, Germany). Electroporation was done according to the manufacturer using 1 pulse 40 ms at 1100 V. After electroporation, cells were cultured in antibiotic free medium on fibronectin/collagen-coated glass cover slips. For patch-clamp or  $\text{Ca}^{2+}$  measurements, transfected cells were identified using anti-CD8 coated dynabeads (Life Technologies GmbH, Darmstadt, Germany).

### ***Patch clamp measurements***

Whole-cell recordings were performed at room temperature on transfected NCI-H295R cells grown on glass cover slips using an EPC-10 amplifier (Heka, Lambrecht, Pfalz, Germany). Patch pipettes with a resistance of 6-10 M $\Omega$  were used for the recordings. The patch pipette solution contained (mM): 95 K-gluconate, 30 KCl, 4.8 Na<sub>2</sub>HPO<sub>4</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 5 glucose, 2.38 MgCl<sub>2</sub>, 0.726 CaCl<sub>2</sub>, 1 EGTA, 3 ATP, pH 7.2. For some experiments, pipette solution contained 30 mM NaCl instead of KCl. The extracellular Ringer-type control solution contained (mM): 140 NaCl, 1.8 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, 10 HEPES, 5 glucose, 5 KCl, pH 7.4. For some experiments, bath Na<sup>+</sup> was replaced by N methyl-D-glucamine chloride (NMDG<sup>+</sup>) to verify the Na<sup>+</sup> permeability of the mutated KCNJ5 channel. To test the effect of the Kir3.4 inhibitor tertiapin-Q and verapamil hydrochloride, conditions were used that allowed detection of wildtype KCNJ5 current: pipette solution with 30 mM Na<sup>+</sup>, and with 0.5 mM ATP replaced by GTP to activate wildtype KCNJ5; bath solution with 50 mM K<sup>+</sup> to increase the inward-current of K<sup>+</sup>.

### ***Ca<sup>2+</sup> measurements***

Intracellular Ca<sup>2+</sup> levels were measured using the ratiometric fluorescent Ca<sup>2+</sup> dye fura-2-AM (Life Technologies GmbH, Darmstadt, Germany). NCI-H295R cells (grown on glass cover slips) were loaded at room temperature for 45 min with 0.5  $\mu$ M fura-2-AM in the presence of 1X Power Load permeabilizing reagent (Life Technologies GmbH, Darmstadt, Germany). The extracellular Ringer-type solution contained (mM): 140 NaCl, 1.8 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, 10 HEPES, 5 glucose, 5 KCl, pH 7.4. For some experiments, bath Na<sup>+</sup> was replaced by NMDG<sup>+</sup>. For stimulation of the cells with 15 mM K<sup>+</sup>, the Na<sup>+</sup> concentration was reduced to the same extent. Mean fluorescence ratios of the emitted light (490-530 nm) after excitation at

340 nm and 380 nm were calculated for single transfected cells from at least 3 different dishes per group using Axiovision software (Zeiss, Jena, Germany).

### ***Substances***

Tertiapin-Q, verapamil hydrochloride, and angiotensin II were purchased from Sigma-Aldrich, (Munich, Germany). Stock solution of verapamil hydrochloride was prepared in DMSO; tertiapin-Q and angiotensin II were dissolved in deionized water.

### ***RNA extraction and gene expression assay.***

Total RNA was isolated from cultured cells using RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. For cDNA generation, 2 µg total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. qPCR was performed in triplicate using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) using TaqMan gene expression assays (Applied Biosystems, Foster City, CA) for *KCNJ5*, nuclear receptor subfamily 4, group A, member 2 (*NR4A2*) and human aldosterone synthase (*CYP11B2*). Gene expression levels were analyzed using the  $2^{-\Delta\Delta CT}$  relative quantification method, using GAPDH as endogenous control.

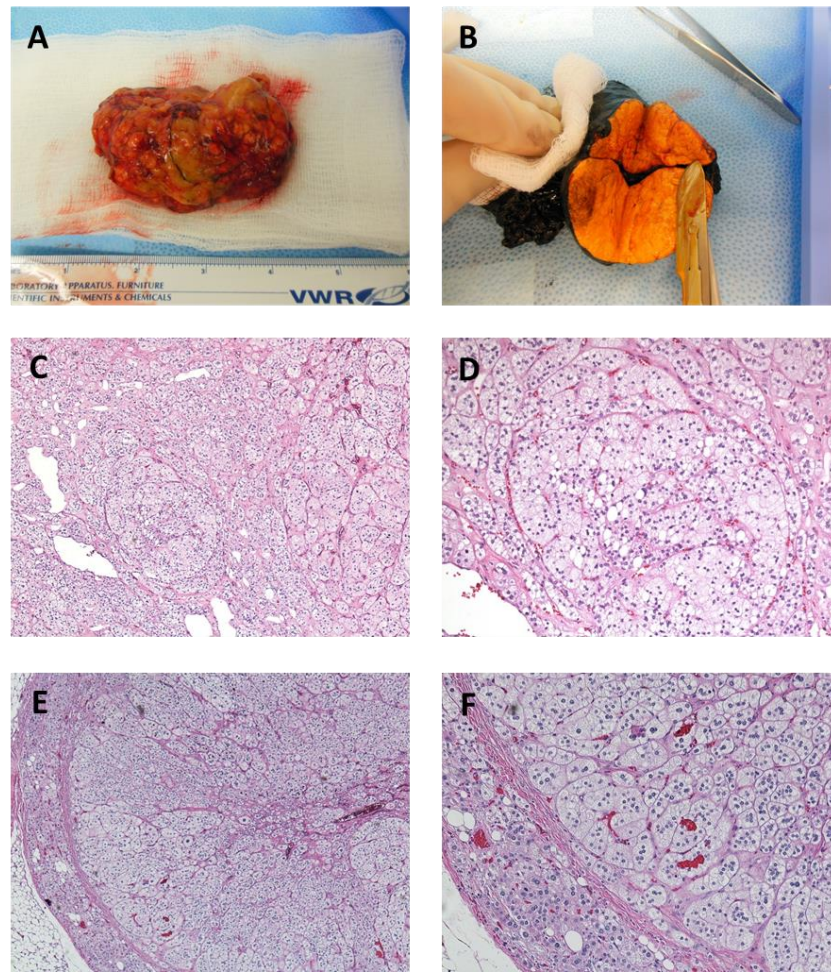
### ***Statistical analyses.***

IBM SPSS Statistics 19 (SPSS INC, Chicago, IL) was used for statistical analyses. Data are expressed as mean  $\pm$  S.D. and individual experiments were repeated at least three times. Differences between variables were evaluated using unpaired *t* test or Mann-Whitney test. A probability of less than 0.05 was considered statistically significant.

### **References.**

1. Fink M, Lesage F, Duprat F, Heurteaux C, Reyes R, Fosset M, Lazdunski M: A neuronal two P domain K<sup>+</sup> channel stimulated by arachidonic acid and polyunsaturated fatty acids. EMBO J. 1998;17:3297-3308.

### Supplementary Figure legends.



### Supplemental Figure S1.

Inspection of the excised adrenals showed markedly enlarged glands (**A**) with homogeneous yellow-orange and lobulated texture (**B**). The right adrenal was 8.5 x 7.0 x 2.5 cm red-tan lobulated adrenal gland with a well-circumscribed prominent mass on the medial superior pole. The cut surface reveals a homogeneous yellow-orange and lobulated texture with a biggest nodule of 3.5 cm in greatest dimension. The cortex measures 0.2 to 1.1 cm and the medulla is up to 0.2 cm in thickness. Left adrenal was a 61 gram, 9.5 x 5.5 x 3.0 cm, red-yellow nodular adrenal gland. The cut surface is yellow-tan and reveals adjacent normal adrenal tissue 1.0 x 0.5 cm and two large nodules 3.0 cm and 4.0 cm in greatest dimensions. The cortex measures 0.5 to 2.5 cm and the medulla is up to 0.2 cm in thickness.

Section of left adrenal gland (**C-D**) shows nodular proliferations of adrenocortical epithelial cells partially circumscribed by a delicate fibrovascular stroma.

Section of right adrenal (**E**) shows a circumscribed nodule composed of sheets of adrenocortical epithelial cells surrounded by a fibrotic capsule adjacent to a rim of uninvolved adrenal cortex (to the left). The higher magnification (**F**) shows the lesion cells of adrenocortical nodule characterized by rounded nuclei with abundant pale vesicular eosinophilic cytoplasm.

			E145 ▼
<b>A</b>	<i>H.s.</i>	SGFVSAFLFSIETETTTIGYGFRVITEKCPEGIILLLV	
	<i>Ma.m.</i>	SGFVSAFLFSIETETTTIGYGFRVITEKCPEGIILLLV	
	<i>C.j.</i>	SGFVSAFLFSIETETTTIGYGFRVITEKCPEGIILLLV	
	<i>L.a.</i>	SGFVSAFLFSIETETTTIGYGFRVITEKCPEGIILLLV	
	<i>O.c.</i>	SGFVSAFLFSIETETTTIGYGFRVITEKCPEGIVLLLV	
	<i>M.m.</i>	SGFVSAFLFSIETETTTIGYGFRVITEKCPEGIILLLV	
	<i>R.n.</i>	SGFVSAFLFSIETETTTIGYGFRVITEKCPEGIILLLV	
	<i>S.s.</i>	SGFVSAFLFSIETETTTIGYGFRVITEKCPEGIVLLLV	
	<i>B.t.</i>	SGFVSAFLFSIETETTTIGYGFRVITEKCPEGIVLLLV	
	<i>G.g.</i>	SGFVSAFLFSIETETTTIGYGYRVITEKCPEGIVLLLI	
	<i>O.h.</i>	SGFVSAFLFSIETETTTIGYGHVITEKCPEGIILLLV	
	<i>X.l.</i>	NGFVSAFLFSIETETTTIGYGYRVITEKCPEGIVLLLV	
			E145 ▼
<b>B</b>	KCNJ5 (Kir3.4)	VENLSGFVSAFLFSIETETTTIGYGFRVITEK	
	KCNJ6 (Kir3.2)	VTNLNGFVSAFLFSIETETTTIGYGYRVITDK	
	KCNJ9 (Kir3.3)	VNNLNGFVAAFLFSIETETTTIGYGHVITDQ	
	KCNJ3 (Kir3.1)	VANVYNFSAFLFFIETETTTIGYGYRYITDK	
	KCNJ1 (Kir1.1)	VENINGLTSAFLFSLETQVTIGYGFRVITEQ	
	KCNJ4 (Kir2.3)	IMHVNGFLGAFLSVETQTTIGYGFRVITEE	
	KCNJ16 (Kir5.1)	VDNVHSFTGAFLSLETQTTIGYGYRCVTEE	
	KCNJ12 (Kir2.2)	VMQVHGFMMAFLFSIETQTTIGYGLRCVTEE	
	KCNJ2 (Kir2.1)	VSEVNSFTAFLFSIETQTTIGYGFRVITEE	
	KCNJ11 (Kir6.2)	VTSIHSFSSAFLFSIEVQVTIGFGGRMVTEE	
	KCNJ10 (Kir4.1)	VVQVHTLTGAFLSLESQTTIGYGFYRISSE	
	KCNJ8 (Kir6.1)	VTNVRSTSAFLFSIEVQVTIGFGGRMTTEE	
	KCNJ15 (Kir4.2)	IMKVDSLTAFLFSLESQTTIGYGVRVITEE	
	KCNJ14 (Kir2.4)	FSHVASFLAFLFALETQTSIGYGVRVITEE	
	KCNJ13 (Kir7.1)	VKYITSFTAFLFSLETQLTIGYGTMFPSGD	
	KCNJ18 (Kir2.6)	VMQVHGFMMAFLFSIETQTTIGYGLRCVTEE	

**Supplemental figure S2.** Multiple allignements of Kir3.4 sequence in different species (**A**) and different members of Kir K<sup>+</sup> channels family (**B**) showing conservation of Glu145. H.s., *Homo sapiens*; Ma. m., *Macaca mulatta*; C.j., *Callithrix jacchus*; L.a., *Loxodonta africana*; O.c., *Oryctolagus cuniculus*; M.m., *Mus musculus*; R.n., *Rattus norvegicus*; S.s., *Sus scrofa*; B.t., *Bos taurus*; G.g., *Gallus gallus*; O.h., *Ophiophagus hannah*; X.l., *Xenopus laevis*.



